

1003 Received 12 MAR 2002

FORM PTO-1390 (REV. 9-2001)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER <div style="text-align: center;">43551</div>	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371					
INTERNATIONAL APPLICATION NO. PCT/FR00/02623		INTERNATIONAL FILING DATE 22 September 2000		PRIORITY DATE CLAIMED 27 September 1999	
TITLE OF INVENTION Transgeneic Animal Expressing a Multiple Mutated Form of Presenilin I					
APPLICANT(S) FOR DO/EO/US Anne Eckert, Wlatter Muller, Chritian Czech, Laurent Pradier and Gunter Tremp					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). <ol style="list-style-type: none"> a. <input checked="" type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 					
Items 11 to 20 below concern document(s) or information included:					
<ol style="list-style-type: none"> 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input checked="" type="checkbox"/> Other items or information: Search Report 					

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 10/088139		INTERNATIONAL APPLICATION NO. PX/T/ΦP00/02623		ATTORNEY'S DOCKET NUMBER 43551	
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<p>21. <input checked="" type="checkbox"/> The following fees are submitted:</p> <p>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</p> <p>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00</p> <p>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00</p> <p>International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00</p> <p style="text-align: center;">ENTER APPROPRIATE BASIC FEE AMOUNT =</p> <p>Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%;">CLAIMS</th> <th style="width: 20%;">NUMBER FILED</th> <th style="width: 20%;">NUMBER EXTRA</th> <th style="width: 20%;">RATE</th> <th style="width: 20%;">\$</th> </tr> <tr> <td>Total claims</td> <td>20 - 20 =</td> <td>0</td> <td>x \$18.00</td> <td>\$</td> </tr> <tr> <td>Independent claims</td> <td>1 - 3 =</td> <td>0</td> <td>x \$84.00</td> <td>\$</td> </tr> <tr> <td colspan="4">MULTIPLE DEPENDENT CLAIM(S) (if applicable)</td> <td>+ \$280.00</td> </tr> <tr> <td colspan="4" style="text-align: center;">TOTAL OF ABOVE CALCULATIONS =</td> <td>\$</td> </tr> <tr> <td colspan="4"> <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. </td> <td>+</td> </tr> <tr> <td colspan="4" style="text-align: center;">SUBTOTAL =</td> <td>\$ 1300.00</td> </tr> <tr> <td colspan="4">Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).</td> <td>\$</td> </tr> <tr> <td colspan="4" style="text-align: center;">TOTAL NATIONAL FEE =</td> <td>\$ 1300.00</td> </tr> <tr> <td colspan="4">Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +</td> <td>\$</td> </tr> <tr> <td colspan="4" style="text-align: center;">TOTAL FEES ENCLOSED =</td> <td>\$</td> </tr> <tr> <td colspan="4"></td> <td>Amount to be refunded:</td> </tr> <tr> <td colspan="4"></td> <td>charged:</td> </tr> </table>				CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	Total claims	20 - 20 =	0	x \$18.00	\$	Independent claims	1 - 3 =	0	x \$84.00	\$	MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+ \$280.00	TOTAL OF ABOVE CALCULATIONS =				\$	<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				+	SUBTOTAL =				\$ 1300.00	Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	TOTAL NATIONAL FEE =				\$ 1300.00	Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$	TOTAL FEES ENCLOSED =				\$					Amount to be refunded:					charged:	CALCULATIONS PTO USE ONLY	
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a. ☒ A check in the amount of \$ 1300.00 to cover the above fees is enclosed.

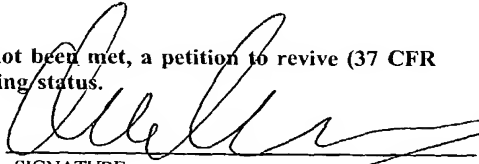
b. ☐ Please charge my Deposit Account No. 18-2220 in the amount of \$ _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
overpayment to Deposit Account No. 18-2220. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card
information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR
1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:


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Initial Information Data Sheet**Inventor Information**

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Application Information

Title Line One::	Transgenic Animal Expressing A Multiple
Title Line Two::	Mutated form of Presenilin 1
Total Drawing Sheets::	11
Formal Drawings?::	No
Application Type::	Utility
Docket Number::	43551

Representative Information

Registration Number::	33,981
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43551

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	:	
	:	
Eckert et al.	:	Patent Art Unit: TBA
	:	
Serial No. Based on PCT/FR00/02623	:	Examiner: TBA
	:	
Filed: 15 March 2002	:	
	:	
For: Transgenic Animal Expressing a Multiple	:	
Mutated Form of Presenilin I	:	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to prosecution on the merits, kindly amend the application as follows:

IN THE SPECIFICATION:

Kindly add the attached page as the new page 35.

IN THE CLAIMS:

3. (Amended) Transgenic animal according to claim 2, characterized in that it allows an apoptotic phenomenon to be detected in its T lymphocytes.
6. (Amended) A method for detecting compounds intended for the treatment of neurodegenerative diseases, comprising exposing said compounds to the animal of any one of claims 1 to 5.
7. (Amended) Cell extracted from the animal of any one of claims 1 to 5.

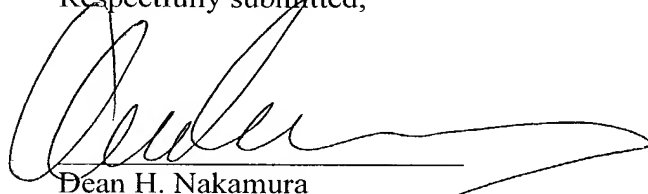
PRELIMINARY AMENDMENT
Serial No. Based on PCT/FR00/02623

Att. Docket No. 43551

8. (Amended) A method for detecting compounds intended for the treatment of neurodegenerative diseases, comprising exposing said compounds to said cell of any one of claims 1 to 5.

Favorable consideration and early indication of allowance are solicited earnestly.

Respectfully submitted,



Dean H. Nakamura
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Dated: 15 March 2002

(12) DEMANDE INTERNATIONALE

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(10) Numéro de publication internationale
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(51) Classification internationale des brevets⁷:

A01K 67/027, C07K 14/47,
C12N 5/10, A61K 49/00, G01N 33/50

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(21) Numéro de la demande internationale:

PCT/FR00/02623

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(22) Date de dépôt international:

22 septembre 2000 (22.09.2000)

(25) Langue de dépôt:

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99/12017 27 septembre 1999 (27.09.1999) FR
60/181,306 9 février 2000 (09.02.2000) US

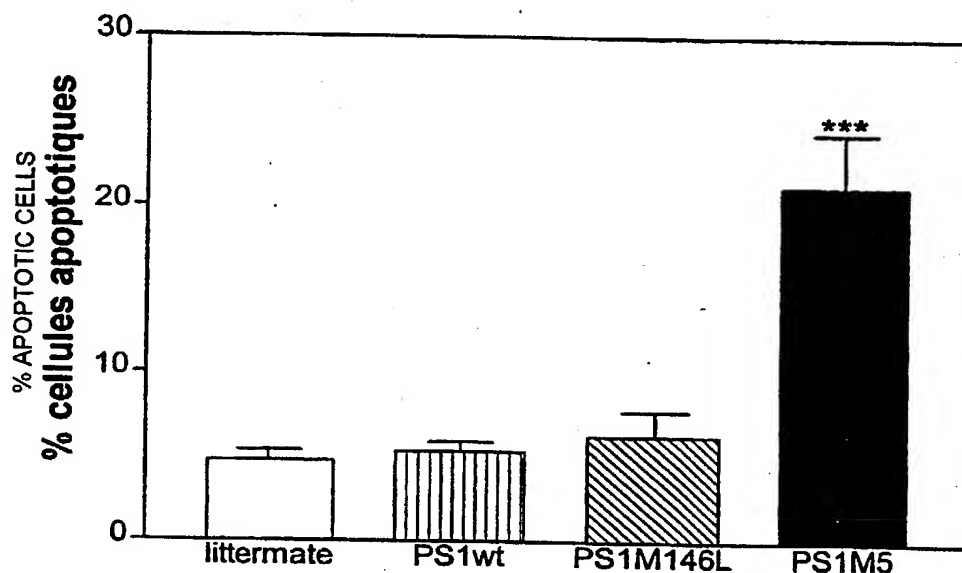
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(81) États désignés (national): AE, AG, AL, AM, AT, AU, AZ,
BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE,

[Suite sur la page suivante]

(54) Title: TRANSGENIC ANIMAL EXPRESSING A MULTIPLE MUTATED FORM OF PRESENILIN 1

(54) Titre: ANIMAL TRANSGENIQUE EXPRIMANT UNE FORME MULTI-MUTÉE DE LA PRESENILINE 1



(57) Abstract: The invention concerns the field of transgenic animal models and more particularly, animal models of Alzheimer disease. The invention relates to a transgenic animal expressing a multiple mutated form of presenilin 1 and enabling to detect an apoptotic phenomenon in a renewable peripheral tissue.

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ST 99042

France

PATENT OF INVENTION

TRANSGENIC ANIMAL EXPRESSING A MULTIMUTATED FORM OF
PRESENILIN 1

RHONE-POULENC RORER S.A.

ABSTRACT

The present invention relates to the field of transgenic animal models and more particularly the animal models of Alzheimer's disease. The invention relates to a transgenic animal expressing a multimutated form of presenilin 1 and allowing an apoptotic phenomenon to be detected in a renewable peripheral tissue.

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CLAIMS

1. Transgenic animal expressing a multimutated form of presenilin 1 and allowing an apoptotic phenomenon to be detected in a renewable peripheral tissue.
2. Transgenic animal according to claim 1, characterized in that it allows an apoptotic phenomenon to be detected in its lymphocytes.
3. Transgenic animal according to [either of] claims [1 and] 2, characterized in that it allows an apoptotic phenomenon to be detected in its T lymphocytes.
4. Transgenic animal according to claim 1, characterized in that the mutations in the PS1 gene are the mutations M146L, H163R, A246E, L286V, C410Y, I143T, L235P, P264L, P267S, E317G, G384A, L392V, A426P and/or P436S.
5. Animal according to claim 4, characterized in that the mutations are M146L, H163R, A246E, L286V, C410Y, combined with each other.
6. [Use of the animal model as described according to claims 1 to 5] ^{A method} ~~for the detection of~~ ^{detecting} compounds intended for the treatment of neurodegenerative diseases [preferably Alzheimer's disease] comprising exposing said compounds to the animal of ~~claims~~ any one of claims 1 to 5.

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7. Cell extracted from ^{The} [an] animal ^[of any one of] [model] as described according to] claims 1 to 5.
8. Use of a cell as described according to claim 7, ^{# A method} for ^{detecting} [the detection of] compounds intended for the treatment of neurodegenerative diseases, [preferably of Alzheimer's disease] ^{comprising exposing said compounds to said cell of any one of claims 1 to 5.}

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11/p8b

ST 99042

France

PATENT OF INVENTION

TRANSGENIC ANIMAL EXPRESSING A MULTIMUTATED FORM OF
PRESENILIN 1

RHONE-POULENC RORER S.A.

ABSTRACT

The present invention relates to the field of transgenic animal models and more particularly the animal models of Alzheimer's disease. The invention relates to a transgenic animal expressing a multimutated form of presenilin 1 and allowing an apoptotic phenomenon to be detected in a renewable peripheral tissue.

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TRANSGENIC ANIMAL EXPRESSING A MULTIMUTATED FORM OF
PRESENILIN 1

The present invention relates to the field of transgenic animal models and more particularly the animal models of Alzheimer's disease. The invention relates to a transgenic animal expressing a multimutated form of presenilin 1 and allowing an apoptotic phenomenon to be detected in a renewable peripheral tissue.

Alzheimer's disease (AD) is a progressive neurodegenerative disease which affects a large proportion of the elderly population. This disease is characterized at the clinical level by a loss of memory and a decline in cognitive functions, and at the neuropathological level by the presence in the brain of intracellular neurofibrillary deposits and extracellular deposits of the β -amyloid ($A\beta$) peptide forming the amyloid plaques (Yanker et al., 1996) as well as a pronounced neuronal loss. In addition to these signs, there are a large number of other abnormal changes including an impairment of the mechanisms of protection against free radicals.

Amyloid plaques are mainly composed of the $A\beta$ peptides containing 40 or 42 residues which are generated during the proteolytic process for the β -amyloid peptide precursor protein (APP). The

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extracellular deposits of A β are very specific for AD and for associated disorders. They represent the early and invariable feature of all the forms of AD, including the familial forms (FAD). The FADs appear relatively early (between 40 and 60 years of age) and are due to mutations in the APP gene in 5% of FAD cases with six identified single or double missense mutations; in the presenilin 1 (PS 1) gene in 50 to 70% of FAD cases with more than 40 different mutations identified to date; and in the presenilin 2 (PS 2) gene in rarer cases of FAD with 2 missense mutations described (for a review see Price and Sisodia, 1998). Mutations in these three genes have been demonstrated to induce changes in the proteolysis of APP, which lead to an overproduction of A β , especially of the long form A β 42, and to the early appearance of the pathology and symptoms which are similar to those of the sporadic forms of AD.

In the transgenic animal models described to date, the symptomatology of neuronal loss comparable to AD is expressed only at the level of the neurons or in their direct vicinity and in particular the phenomenon of apoptosis (Chiu et al., 1999). However, these models have several disadvantages including in particular the need to breed a very large number of animals, most often over long periods of time, which may be up to 24 months, in order to monitor the appearance of the

symptoms of AD, the systematic sacrificing of the animals to study the pathology and thus experimental protocols which are particularly tedious and expensive.

No animal model of AD therefore exists which makes it possible to measure the symptoms and, in particular, the phenomena of cell death associated with AD in peripheral tissues.

The present invention therefore results from the search for a new animal model representative of the neuropathology which makes it possible to measure the symptoms associated with AD, and in particular apoptosis, in peripheral tissues.

A first subject of the invention therefore relates to a transgenic animal model of Alzheimer's disease expressing multimutated presenilin 1.

Transgenic animal is understood to mean any nonhuman animal exhibiting a modification of its genome. The modification of the genome may result from an impairment or a modification of one or more genes by "knock-in" or by "knock-out". This modification may be due to the action of conventional impairing or mutagenic agents or may be performed by site-directed mutagenesis, as is described in Materials and Methods.

The modification of the genome may also result from an insertion of a gene or of genes or from replacement of a gene or of genes in its (their) wild-type or mutated form.

The sequence of the nonmutated human PS1 protein was described by Sherrington et al in 1995. Multimutated protein is understood to mean the PS1 protein comprising at least three mutations which are combined or associated with each other, that is to say which are present at the same time in the said protein. According to a preferred embodiment of the invention,

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the DNA encodes the PS1 gene which comprises 5 mutations (PS1M5).

The mutations in the PS1 gene may be one of the 40 mutations described to date in the literature. Preferably, the mutations in the PS1 gene are M146L, H163R, A246E, L286V, C410Y, I143T, L235P, P264L, P267S, E317G, G384A, L392V, A426P and/or P436S. They are in partial combination with each other.

The mutations M146L, H163R, A246E, L286V, C410Y, combined with each other, are preferred for producing a model according to the invention.

In the context of the model of the invention, the DNA is placed under the control of sequences allowing its expression and in particular of transcription promoter sequences.

As promoter sequences, there may be mentioned most particularly the HMG promoter (Gautier et al., 1989), as well as the PDGF promoter (Sasahara et al., 1991), the Thy-1 promoter (Lüthi et al., 1997) and the Prion gene promoter (Scott et al., 1992).

According to a particularly advantageous embodiment of the invention, the animal model comprises the PS1 gene having the M146L, H163R, A246E, L286V, C410Y mutations, placed under the control of the HMG promoter.

The animal model according to the invention is very advantageous because it corresponds to a

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practical model which is representative of the phenomena of cell death in AD. Indeed, this model exhibits symptoms associated with AD including in particular apoptosis of the cells and oxidative stress and makes it possible, in addition, to measure these symptoms in the cells of renewable peripheral tissues. It should be noted that oxidative stress also manifests itself in the brain of these animals. Renewable peripheral tissues should be understood to mean any tissue exhibiting a renewal of these cells over time. By way of example of renewable peripheral tissue, there may be mentioned the spleen, the liver, blood and the like. Preferably, the apoptotic phenomenon is measured in blood cells and still more preferably in the lymphocytes. Among the lymphocytes, the T lymphocytes are preferred for the invention.

Thus, the results described in the examples demonstrate that the transgenic mouse expressing the multimutated PS1 develops cellular impairments which are found in Alzheimer's disease and, in particular, exhibits increased sensitivity to apoptosis. This phenomena is moreover not observed with a simple natural pathological mutant of the M146L type. This phenotype is specifically obtained with a nonnatural form grouping together several individual mutations, and preferably 5 mutations, on the same cDNA. In addition, through the ectopic expression of the

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transgene by virtue of the ubiquitous promoter, this model makes it possible to detect an apoptotic phenomenon (linked to the mutations in Alzheimer's disease) in a renewable peripheral tissue. This model therefore provides a much more practical source of material (not requiring sacrificing the animal), therefore allowing longitudinal monitoring.

Furthermore, the impairments of the metabolism of calcium and of the free radicals which are observed very clearly in this model are similar to the increase in the latent period for the calcium response and the oxidative stress which are observed with Alzheimer patients (Eckert et al., 1997 and 1998), which reinforces the relevance of this model.

The present invention therefore also relates to the use of the animal model, as described above, for the detection of compounds intended for the treatment of neurodegenerative diseases, preferably Alzheimer's disease.

Indeed, through its advantageous properties, this model allows, in comparison with known models, the detection of compounds which are particularly suitable for the treatment of AD, in particular as described in humans.

These compounds may be chemical molecules, peptide or protein molecules, antibodies, chimeric molecules as well as antisense DNAs or ribozymes.

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The compounds which are detected may be used as a medicament, as they are or in combination with a pharmaceutically acceptable vehicle in order to obtain a pharmaceutical composition. This may include in particular isotonic, sterile saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like, or mixtures of such salts), or dry, in particular freeze-dried, compositions which, upon addition, depending on the case, of sterilized water or of physiological saline, allow the constitution of injectable solutions. The injections may be carried out by the stereotaxic, topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular or transdermal route, and the like.

The detection of the compounds described above is based on bringing the animal model of the invention into contact, especially by an administration such as for example an injection, with a compound or a mixture of compounds presumed to have an action and then measuring the effect(s) of the compounds in particular at the level of the peripheral tissues of the model on the different biochemical and/or histological changes such as for example those described in the Methods and Results section including apoptosis, the level of intracellular calcium, the level of free radicals and the like.

Another subject of the invention relates to a cell extracted from the animal model as described above as well as its use for the detection of compounds intended for the treatment of neurodegenerative diseases, preferably Alzheimer's disease.

The detection of compounds described above is based on bringing cells extracted from the animal model of the invention into contact with a compound or a mixture of compounds presumed to have an action and then measuring the effect(s) of the compounds at the level of the whole cells, in cell homogenates or on a subcellular fraction, on different parameters such as cell death, the production of the A β peptide, production of free radicals, and the like.

The results described in the examples demonstrate the advantages of the model of the invention and clearly support the use of this transgenic model as a simple and rapid measuring and monitoring tool in the context of therapeutic strategies such as in particular the development of antiapoptotic agents or of agents limiting cell death linked to AD more generally.

The present invention will be described in greater detail with the aid of the examples which follow but which should be considered as illustrative and nonlimiting.

LEGEND TO THE FIGURES

Figure 1: Analysis of the expression of human PS1 in the transgenic mice PS1M5 (lanes 1,5,9), PS1M146L (lanes 2,6,10), PS1wt (lanes 3,7,11) and non.transgenic (lanes 4,8,12). The transgenic mouse tissues: lymphocytes (lanes 1-4), spleen (lanes 5-8) and brain (lanes 9-12), were lysed and analysed by immunoblotting using an antibody specific for the human sequence of PS1 (epitope in the N-term region of PS1). The holoprotein PS1 (approx. 50 kDa) as well as the N-terminal fragment are observable. The expression of the transgene is observable for the three transgenic mice. There is absence of endoproteolytic cleavage for the protein PS1M5.

Figure 2: Increased apoptosis of the lymphocytes derived from transgenic mice PS1M146L and PS1M5 under basal conditions. The levels of apoptosis under basal conditions were measured in dissociated lymphocytes. The PS1M5 lymphocytes show a higher level of apoptosis compared with PS1wt (*, $p < 0.05$) or with the nontransgenic littermate controls (**, $p < 0.05$). The same applies to the PS1M146L lymphocytes compared with the littermate controls (+, $p < 0.05$).

Figure 3: Apoptosis after 2.5 h of incubation of lymphocytes derived from transgenic mice. After 2.5 h of incubation, the PS1M5 lymphocytes show a significantly higher level of apoptosis (***, $p < 0.001$) compared with PS1wt, PS1M146L or with the nontransgenic controls (littermates).

Figure 4: Apoptosis induced by deoxyribose treatment of lymphocytes derived from transgenic mice. After induction with deoxy-D-ribose (10 mM), the apoptosis levels are significantly higher in the PS1M5 group than in the other groups (**, $p < 0.01$ vs PS1wt and PS1M146L; ***, $p < 0.001$ vs littermates).

Figure 5: Apoptosis induced by hydrogen peroxide treatment of lymphocytes derived from transgenic mice. After induction with hydrogen peroxide (1 mM), the apoptosis levels are significantly higher in the PS1M5 group than in the other groups (***, $p < 0.001$). There is no difference between the other groups.

Figure 6: Apoptosis induced by dexamethasone treatment of lymphocytes derived from transgenic mice. After induction with dexamethasone (10^{-7} M), the apoptosis levels are significantly higher in the PS1M5 group than in the other groups (*, $p < 0.05$ vs PS1M146L and

littermates; **, $p < 0.01$ vs PS1wt). There is no difference between the other groups.

Figure 7: Increase in the levels of free radicals in the lymphocytes of transgenic mice PS1M5. The levels of the oxygenated radical species (Reactive Oxygen Species) were measured in the mouse lymphocytes by flow cytometry (Rhodamine 123) and expressed as mean fluorescence intensity (MFI). The levels of ROS are significantly higher in the lymphocytes of transgenic mice PS1M5 compared with the other groups (*, $p < 0.05$ vs PS1wt; **, $p < 0.01$ vs littermates).

Figure 8: Increased mobilization of intracellular calcium in the lymphocytes of transgenic mice PS1M5. The levels of $[Ca^{2+}]_i$ were determined under resting (basal) conditions. The levels of intracellular calcium are higher in the lymphocytes of transgenic mice PS1M5 than in the other groups. There is no difference between the other groups.

Figure 9: Increased intracellular calcium response after stimulation with PHA in the lymphocytes of transgenic mice PS1M5. The difference between the levels of $[Ca^{2+}]_i$ after and before mitogenic stimulation with PHA (15 microg/ml) has been represented. The lymphocytes of transgenic mice PS1M5 respond more

strongly to the stimulus than the other groups ($p < 0.01$). There is no difference between the other groups.

Figure 10: Latency for the slower mobilization of intracellular calcium in the lymphocytes of transgenic mice PS1M5 and PS1M146L. The time interval to reach the peak for $[Ca^{2+}]_i$ after mitogenic stimulation with PHA is higher in the lymphocytes of transgenic mice PS1M5 (**, $p < 0.05$ vs PS1wt and littermates) as well as to a lesser degree for the transgenic mice PS1M146L (*, $p < 0.05$ vs PS1wt). There is no difference between the mouse PS1wt and the nontransgenic controls (littermates).

Figure 11: Effect of the human mutations of PS1 in the brain of transgenic mice on the mechanisms of protection of the free radicals. A) Levels of activity of the enzyme superoxide dismutase, SOD. B) Levels of activity of the enzyme glutathione reductase. C) Levels of lipid peroxidation after stimulation with $FeCl_3$. A significant reduction in the mechanisms of detoxification of the free radicals (SOD and GR) is observed in the brain of the transgenic mice PS1M5 (*, $p < 0.05$ vs PS1wt; **, $p < 0.05$ vs PS1M146L) with a tendency towards reduction in the mice PS1M146L. Conversely, the levels of stimulated lipid peroxidation

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(due to the presence of free radicals) is increased in the mice PS1M5.

MATERIALS AND METHODS

1. Mutagenesis of presenilin 1-PS1

The human cDNA for PS1 containing a Kozak consensus at the level of the initial ATG has been previously described (Pradier et al., 1999). The mutagenesis of PS1 was carried out using the SculptorTM in vitro mutagenesis kit (Amersham, France). The coding region of PS1 was subcloned into the vector Bluescript (stratagene) and a single-stranded DNA prepared. The 5 mutations used in the context of the invention were introduced with the aid of oligonucleotides containing the desired mutations according to the supplier's instructions:

M146L, 5'GAGGATAGTCG*TGACAACAAT³'; SEQ ID No. 1

H163R, 5'AAGCCAGGCC*TGGATGACCTT³'; SEQ ID No. 2

A246E, 5'GATGAGCCAT*GCAGTCCATTG³'; SEQ ID No. 3

L286V, 5'GGAGTAAATGC*GAGCTGGAAA³'; SEQ ID No. 4

C410Y, 5'GGCTACGAAT*CAGGCTATGGT³'; SEQ ID No. 5

the * denotes the position of the nucleotide mutation introduced on the complementary strand. To obtain the construct containing the five combined mutations (PS1M5 for mult mutant5), five successive mutageneses were carried out. The entire sequence of the PS1 cDNA was

checked on each mutagenesis to ensure the absence of undesirable mutations.

2. Generation and identification of the transgenic mice

For the construction of the transgenes, the cDNAs for PS1 wild type, PS1M146L and PS1M5 were subcloned into the SmaI/BamHI restriction sites of the multiple cloning site of the transgenic expression vector HMG (Czech et al., 1997). The cDNAs are under the control of the HMG-CoA reductase partial promoter which allows ubiquitous expression of the transgene. For the microinjection, the expression cassette was purified by gel electrophoresis after restriction with the enzyme NotI in order to eliminate the unimportant sequences from the vector. The purified transgene was taken up at the final concentration of 2.5 ng/microl in 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA and injected into one of the two pronuclei of fertilized mouse oocytes. The surviving embryos are transplanted into the oviduct of an adoptive mother. The presence of the transgene was analysed by PCR and Southern. The PCR was carried out using oligonucleotides specific for the human PS1 sequence having the sequences SEQ ID No. 6

5'-TAA TTG GTC CAT AAA AGG C-3' and SEQ ID No. 7

5'-GCA CAG AAA GGG AGT CAC AAG-3', generating an amplification fragment of 550 bp. For the Southern

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analysis, a 1.2 kb PstI-SalI fragment, corresponding to the first intron of the HMG expression cassette was labeled with alpha-³²P and used as probe for the detection of the transgene and of the endogenous HMG-CoA reductase gene. By virtue of the latter analysis, the absence of any major rearrangement or of any deletion within the transgene may be guaranteed. The mice were bred in accordance with the French rules for caring for animals.

3. Immunoblotting

The cerebral tissue and the spleen of transgenic mice (PS1wt, PS1M146L and PS1M5) and of nontransgenic control mice (littermate) were homogenized on ice in a 0.32 M sucrose solution containing protease inhibitors (CompleteTM, Boehringer-Mannheim, Germany). The cellular debris were removed by centrifugation at 4°C for 5 min at 1500 g. The lymphocyte lysates were prepared in the same manner from the fraction of purified cells. The protein concentration in the supernatant was measured with the aid of the BCA protein test (Pierce, USA). For the detection of PS1, 25 µg of protein extract were incubated at 56°C for 20 min in the Laemmli loading buffer containing 8M urea and 50 mM dithiothreitol. The proteins were fractionated by polyacrylamide gel electrophoresis (SDS-PAGE). After transferring the

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proteins onto nitrocellulose filter (Amersham, France) the filter was heated in PBS for 5 min in order to increase the sensitivity and immediately saturated with 5% (w/V) of powdered skimmed milk in TBS 50 mM Tris-HCl pH 8.1, 150 mM NaCl, 0.05% (V/V) Tween 20 for 1 h and incubated overnight at 4°C with the human anti-PS1 primary antibody (Ab), MAB1563 (Chemicon, USA), diluted 1/10,000 in TBS buffer alone. The binding of the Ab was detected with an anti-IgG Ab conjugated with horseradish peroxidase (Amersham, France) followed by a chemiluminescence detection system (Amersham, France) according to the manufacturer's instructions.

4. Preparation of the lymphocytes

The lymphocytes were prepared from freshly dissociated mouse spleen. The spleen is homogenized in RPMI buffer and then the homogenate is passed through a 10-micron filter. The cellular homogenate is washed several times by centrifugation and resuspension. After lysis of the erythrocytes present, the number of cells is determined by counting under a microscope.

To obtain the T lymphocytes, the B cells were counterselected by attachment onto magnetic beads carrying anti-B cell antibodies (Dynabeads Mouse pan B, Dynal, Norway) and separation of the beads. The remaining cells are T lymphocytes (CD3-positive) at

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more than 80% as determined by flow cytometric analysis.

5. Measurement of apoptosis

To determine the content of DNA at the G1 phase, which defines the percentage of apoptotic cells, after treatment at various times, the T lymphocytes are separated by centrifugation and the cellular pellets taken up in lysis buffer (0.1% sodium citrate, 0.1% Triton X-100) containing 50 µg/ml of propidium iodide (Sigma, Munich). The samples are stored at 4°C for 1-2 hours before flow cytometric analysis (FACSCalibur, Benckton Dickinson, Cell Quest software). Cell death was induced by various treatments: 2-deoxy-D-ribose (d-Rib, 10mM), hydrogen peroxide (H₂O₂, 1 mM) and dexamethasone (dex, 10⁻⁷ M) for 2.5 h. Cell death determined in the absence of treatment was defined as *in vitro* spontaneous apoptosis.

6. Measurement of intracellular calcium

The T cell fraction is taken up in RPMI buffer and incubated in the presence of Fura-2-AM (Molecular Probe, Leiden, The Netherlands). The incubation is terminated by addition of HBSS buffer and washing of the suspension by centrifuging several times in order to remove the dye from the medium. The T cell

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fraction is finally taken up in HBSS buffer and stored on ice until the measurement of intracellular calcium.

The Fura-2 fluorescence was measured as previously described (Eckert et al., 1993) using an SLM-Aminco luminescence spectrometer with wavelengths of excitation at 340 nm and 380 nm and of emission at 510 nm. The intracellular concentration of calcium $[Ca^{2+}]_i$ was calculated from the method of ratios of Grynkiewicz as previously described (Eckert et al., 1997) using a K_d value of 224 nM. As stimulator of calcium mobilization in the lymphocytes, PHA-P (Sigma, Munich) was added at the concentration of 15 micrograms/ml.

7. Measurement of the free radicals

The production of free radicals (ROS) was quantified by flow cytometry (FACSCalibur, Benckton Dickinson) using dihydrorhodamine-123 (DHR, Molecular Probes) as fluorescent revealing agent. The cells are resuspended in 1 ml of HBSS buffer in the presence of DHR (final concentration 10microm) and incubated at 37°C for 15 min on a shaking bath. The conversion of DHR to its fluorescent derivative rhodamine-123 is then quantified and expressed as mean fluorescence intensity (MFI).

8. Preparation of the cerebral tissue

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The mice were used at the age of 3-4 months and sacrificed by decapitation. The brains were removed and washed extensively in buffer on ice. The tissue (cerebellum included) was weighed and immediately frozen at -20°C . The tissue was homogenized with the aid of a Potter-Elvehjem homogenizer in Tris-HCl buffer at 5 and 20 mM, respectively, in order to obtain homogenates diluted (weight/volume) 1/10 and 1/5 respectively.

9. Test of CuZn SOD and glutathione reductase activity

The brain homogenates (1/5 w/v) were centrifuged at $8500 \times g$ for 10 minutes at 4°C . The supernatant was used to measure the SOD and glutathione reductase (GS) activities. The SOD activity was measured with the aid of an SOD-525 type kit (Calbiochem, Germany). This kit uses a specific reagent (R1) which undergoes alkaline auto-oxidation which is accelerated by superoxide dismutase. A second reagent (R2) is used to eliminate the interference caused by mercaptans, such as glutathione. One unit of SOD-525 activity is defined as the activity doubling the level of auto-oxidation of R1. The GR activity was similarly assayed with the aid of a specific kit (Calbiochem, Germany). This kit measures the level of oxidation of NADPH to NADP^{+} which is accompanied by a reduction in

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absorbance at 340 nm which is detected by spectrophotometry. One unit of GR activity is defined by the reduction of one micromole of GSSG at 25°C, pH 7.6.

10. Measurement of the basal and stimulated lipid peroxidation (LPO) in the cerebral tissues

The brain homogenates (1/10, w/v) are incubated in buffer containing (stimulated LPO) or otherwise (basal LPO) FeCl_3 and 100 μM for 30 min at 37°C in a stirred aqueous bath. After incubation, the homogenates are centrifuged at 3000 \times g for 10 min. The supernatants are used to detect lipid peroxidation by measuring the concentration of malondialdehyde (MDA) with the aid of an LPO-586 type kit (Calbiochem). This kit uses a specific chromophore which reacts with MDA at moderate temperature (45°C).

EXAMPLES

Example 1: Immunodetection of the proteins

corresponding to the transgenes PS1wt, PS1M146L and PS1M5

Transgenic mice PS1wt, PS1M146L and PS1M5 (multimutant) were generated. The transgene is under the control of the human HMG-CoA reductase promoter, a housekeeping gene which confers a high ubiquitous expression including the brain (Gautier et al., 1989,

Czech et al., 1997 and 1998). Analysis of the levels of expression of the transgenes (human PS1) was carried out on the spleen and brain tissues as well as on the lymphocytic cell fraction with the aid of an antibody specific for the human PS1 sequence not recognizing the mouse homologue (Fig. 1). In the brain (Fig. 1, lanes 9-12), the N-terminal fragment of PS1 is the predominant species with higher levels for PS1M146L compared with PS1wt. For PS1M146L, the holoprotein (complete protein, approx. 50 kDa) is detectable, demonstrating a saturation of the endoproteolysis process at high expression of PS1 as previously described. On the other hand, for PS1M5, the multimutant, there is complete absence of endoproteolysis and only the holoprotein is detectable. Similar results were observed in the spleen and the lymphocytes with variable but detectable levels of expression in all these tissues.

Example 2: Enhanced spontaneous apoptosis in the lymphocytes isolated from multimutated PS1 transgenic mice

The aim of this example is to demonstrate that the peripheral cells of the animal model expressing the multimutated PS1 exhibits an enhanced spontaneous apoptosis which is persistent over time.

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The lymphocytes isolated from transgenic mice expressing PS1wild type (without mutation) have the same level of basal apoptosis (2.8%) as the nontransgenic controls derived from the same litter (littermate) (Fig. 2). On the other hand, this spontaneous apoptosis is greatly increased (6%) in the transgenic mice expressing the mutation PS1-M146L or the multimutation PS1M5.

Very interestingly, after culturing for 2.5 h without apoptotic stimulus, the levels of spontaneous apoptosis for the lymphocytes of PS1M5 were higher than for PS1wt or the simple mutant PS1M146L or the nontransgenic controls (Fig. 3).

These results demonstrate that the cells of the transgenic animal of the invention and in particular those of the peripheral tissues (lymphocytes in particular) exhibit not only a greater spontaneous apoptosis compared with the controls but also that it persists over time.

Example 3: Increase in apoptosis induced by treatment with deoxyribose in the lymphocytes derived from PS1M5 transgenic mice

The aim of this example is to demonstrate the apoptosis induced by deoxyribose is much greater in the multimutant PS1 mouse model.

After induction by treatment with 2-deoxy-D-ribose (d-Rib), the levels of apoptosis are significantly greater for the transgenic lymphocytes PS1M5 than for PS1wt, simple mutant PS1-M146L or the nontransgenic controls (Fig. 4).

This increase in apoptosis after induction demonstrates an enhanced response (sensitization) to an apoptotic stress due to the expression of multimutant PS1. Combined with an increase in basal apoptosis, these results clearly indicate that the transgenic expression of a multimutated form of PS1 causes a much higher sensitivity to apoptosis in the lymphocytes and the like.

Example 4: Increase in apoptosis induced by treatment with hydrogen peroxide in the lymphocytes derived from PS1M5 transgenic mice

The aim of this example is to demonstrate that the apoptosis induced by hydrogen peroxide is much greater in the multimutant PS1 mouse model.

After stimulation by treatment with hydrogen peroxide (H2O2), the levels of apoptosis are significantly greater in the lymphocytes PS1M5 than for the others which are transgenic or nontransgenic ($p < 0.001$, Fig. 5). There is no significant difference between the transgenic lymphocytes PS1wt (S182) and simple mutant PS1M146L or the nontransgenic controls.

Example 6: Increase in the levels of free radicals in the lymphocytes of PS1M5 transgenic mice

The levels of oxygenated free radical agents (Reactive Oxygen Species) were measured in the mouse lymphocytes by flow cytometry. The ROS levels are significantly higher in the lymphocytes of transgenic mice PS1M5 ($p < 0.05$) compared with the other groups (Fig. 7). There is no significant difference between the other groups although a trend towards an increase in the ROS levels appears between the littermate controls and the transgenics PS1wt and PS1M146L.

This increase in the ROS levels demonstrates that it is possible to detect, under basal conditions, in this model an impairment of a biochemical parameter (greatly affected in AD) which may underlie the hypersensitivity to apoptosis.

Example 7: Enhanced mobilization of intracellular calcium in the lymphocytes of PS1M5 transgenic mice

Because apoptosis is modulated by the intracellular levels of calcium, these were analysed in the lymphocytes of the transgenic mice. At rest, the levels of $[Ca^{2+}]_i$ for the controls and the transgenic mice PS1wt and PS1M146L are identical (less than or equal to about 150 nM). The multiple mutant PS1M5 demonstrates a slight rise in the basal levels (greater than 200 nM) (Fig. 8).

After mitogenic stimulation (PHA, 15 microg/ml), the levels of intracellular calcium increase by approximately 70 nM in the control mice and the transgenic mice PS1wt and PS1M146L (Fig. 9). On the other hand, for the mice PS1M5, this increase is 190 nM which is statistically different from the other groups ($p < 0.01$). Therefore, not only are the levels of $[Ca^{2+}]_i$ higher under basal conditions, but the clear response to a stimulus is also greater, resulting in much higher absolute levels (400 nM compared with 200-220 nM for the other groups, statistically significant difference).

Furthermore, compared with the nontransgenic controls which exhibit a very rapid calcium response, the latent period to reach the $[Ca^{2+}]_i$ peak after stimulation with PHA is greatly increased in the lymphocytes of the PS1M5 transgenics and to a lesser degree for the PS1M146Ls (Fig. 10) but not in the PS1wts.

The differences in the levels and kinetics of calcium responses in the lymphocytes demonstrate a substantial alteration of the processes of mobilization of the intracellular calcium reserves due to the expression of multimutant PS1 in this model.

The alteration of the kinetics of the calcium responses had been previously demonstrated in the

lymphocytes of AD patients, which reinforces the relevance of this animal model.

Example 8: Study of the metabolism of the free radicals in the brain

To confirm the relevance, relative to Alzheimer's disease, of the deficiencies observed in the transgenic mice of the invention, it was investigated if pathological impairments were identifiable in the brain of these animals. In particular, the mechanisms for protection against free radicals were analysed since the latter are involved in the apoptosis phenomena linked to the presenilins and since an impairment in the lymphocytes of PS1M5 transgenic mice was demonstrated in Example 6.

The analyses were carried out on 3-4-month-old animals. Compared with the transgenic mice PS1wt, the PS1M146Ls demonstrate a reduction in the levels of SOD activity by approximately 20% in the brain (Fig. 11A). This reduction is further accentuated in the PS1M5 transgenics (-28%, $p < 0.05$).

The glutathione reductase activity is also significantly reduced in the brain of the PS1M5 transgenics (-27%, $p < 0.05$). By contrast, a truly modest reduction in this activity modest effect is noted in the PS1-M146L transgenics (Fig. 11B).

The basal lipid peroxidation levels were identical in all the groups of mice. After stimulation with FeCl_3 , on the other hand, the lipid peroxidation levels are increased in the PS1M5 transgenics (+20%, Fig. 11C).

In multimitated PS1M5 transgenic young adults (3-4 months), a deficiency is therefore observed in the mechanisms for protection from free radicals and in parallel an increase in sensitivity to lipid peroxidation in the brain. This effect perfectly correlates with the increased sensitivity to apoptosis, the impairments in the mobilization of intracellular calcium and the increase in the levels of oxygenated free radical species observed in the lymphocytes of these transgenics which artificially express the transgene in these two tissues. The deficiency in the mechanisms for protection against free radicals was also revealed in patients suffering from Alzheimer's disease, thus confirming the relevance of this animal model.

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CLAIMS

1. Transgenic animal expressing a multimutated form of presenilin 1 and allowing an apoptotic phenomenon to be detected in a renewable peripheral tissue.
2. Transgenic animal according to claim 1, characterized in that it allows an apoptotic phenomenon to be detected in its lymphocytes.
3. Transgenic animal according to either of claims 1 and 2, characterized in that it allows an apoptotic phenomenon to be detected in its T lymphocytes.
4. Transgenic animal according to claim 1, characterized in that the mutations in the PS1 gene are the mutations M146L, H163R, A246E, L286V, C410Y, I143T, L235P, P264L, P267S, E317G, G384A, L392V, A426P and/or P436S.
5. Animal according to claim 4, characterized in that the mutations are M146L, H163R, A246E, L286V, C410Y, combined with each other.
6. Use of the animal model as described according to claims 1 to 5 for the detection of compounds intended for the treatment of neurodegenerative diseases, preferably Alzheimer's disease.

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7. Cell extracted from an animal model as described according to claims 1 to 5.

8. Use of a cell as described according to claim 7, for the detection of compounds intended for the treatment of neurodegenerative diseases, preferably of Alzheimer's disease.

to use for the detection of compounds intended for the treatment of neurodegenerative diseases, preferably of Alzheimer's disease.

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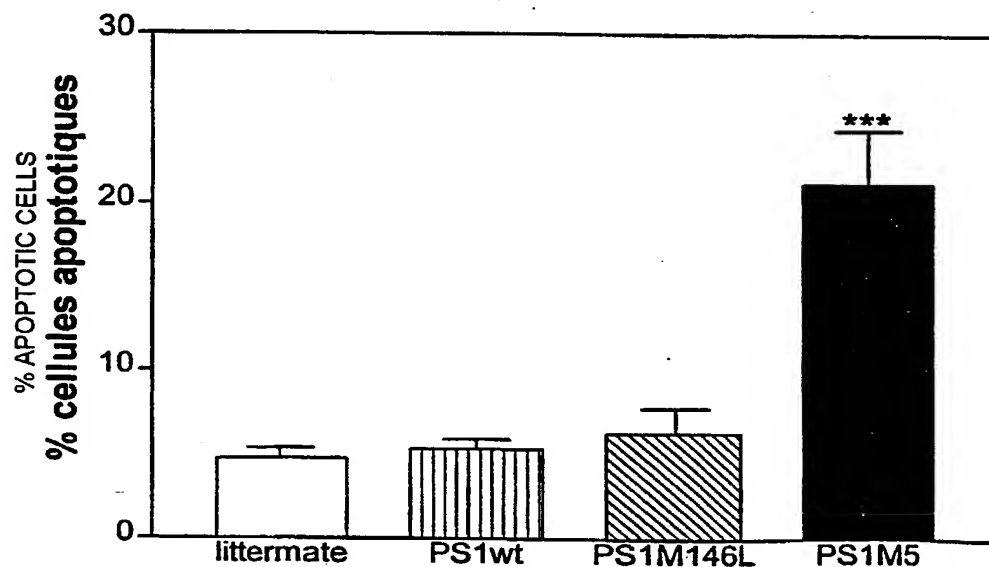
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(54) Title: TRANSGENIC ANIMAL EXPRESSING A MULTIPLE MUTATED FORM OF PRESENILIN 1

(54) Titre: ANIMAL TRANSGENIQUE EXPRIMANT UNE FORME MULTI-MUTÉE DE LA PRESENILINE 1



(57) Abstract: The invention concerns the field of transgenic animal models and more particularly, animal models of Alzheimer disease. The invention relates to a transgenic animal expressing a multiple mutated form of presenilin 1 and enabling to detect an apoptotic phenomenon in a renewable peripheral tissue.

(57) Abrégé: La présente invention concerne le domaine des modèles animaux transgéniques et plus particulièrement, les modèles animaux de la maladie d'Alzheimer. L'invention se rapporte à un animal transgénique exprimant une forme multi-mutée de la préséniline 1 et permettant de détecter un phénomène apoptotique dans un tissu périphérique renouvelable.

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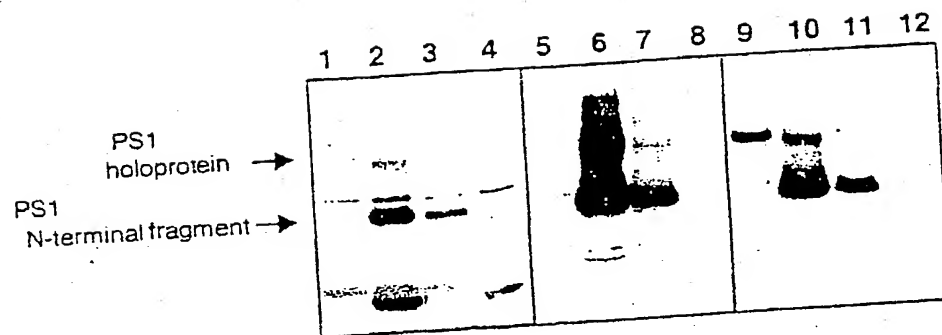


Figure 1

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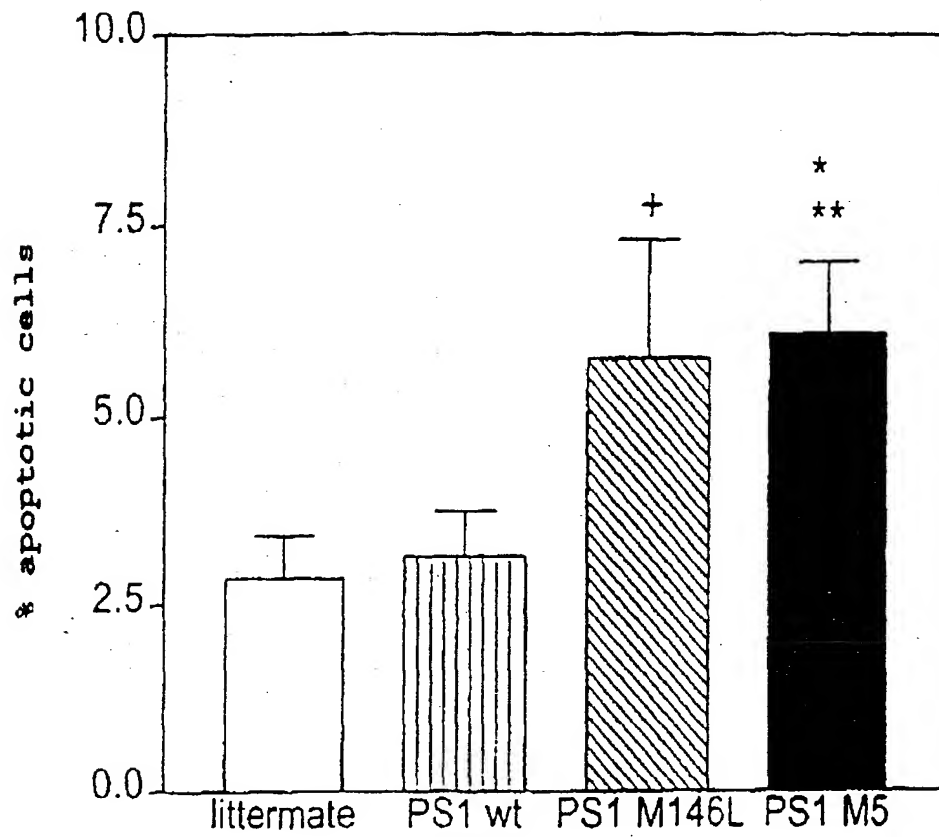


Figure 2

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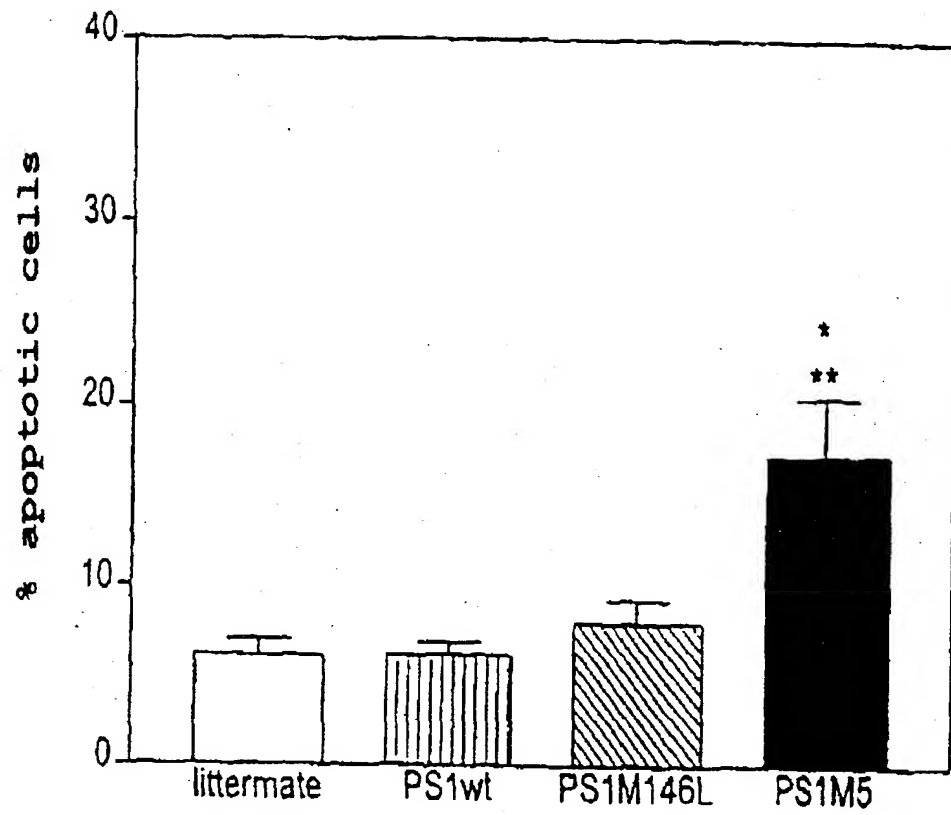


Figure 3

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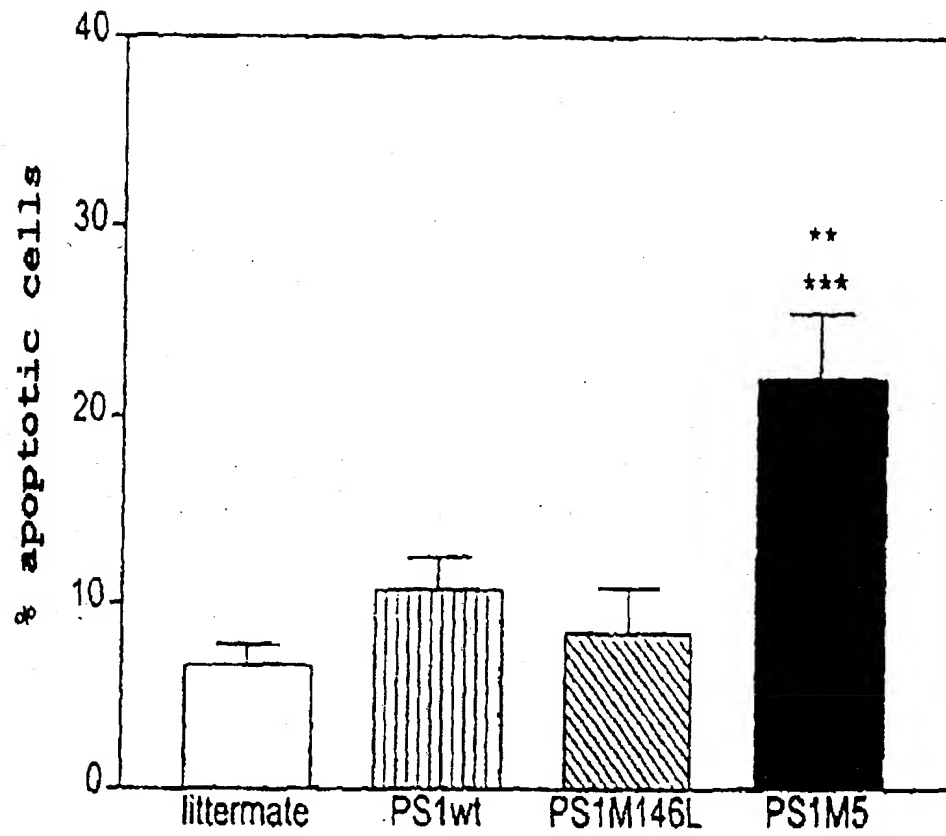


Figure 4

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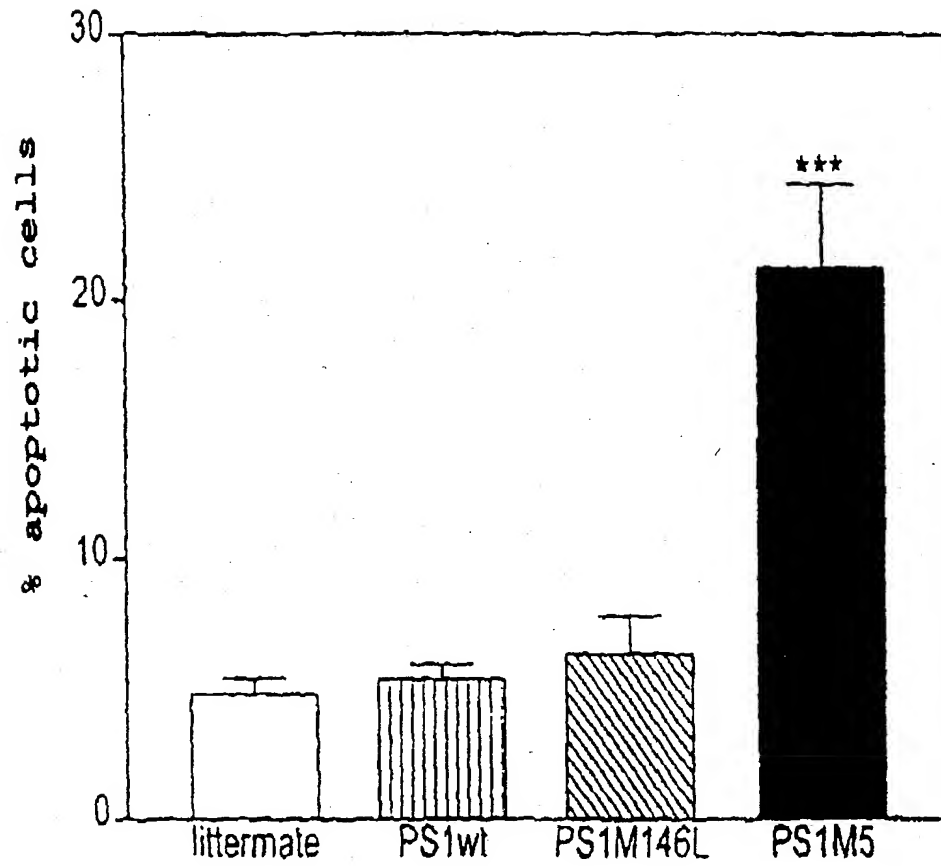


Figure 5

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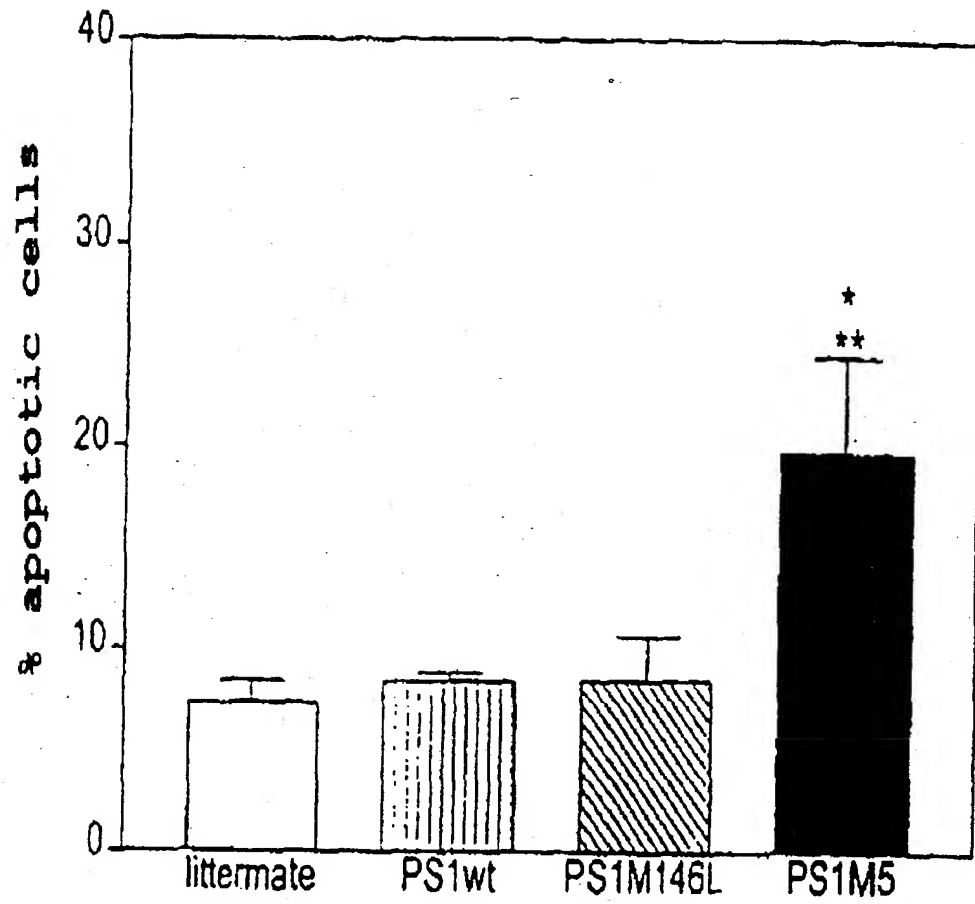


Figure 6

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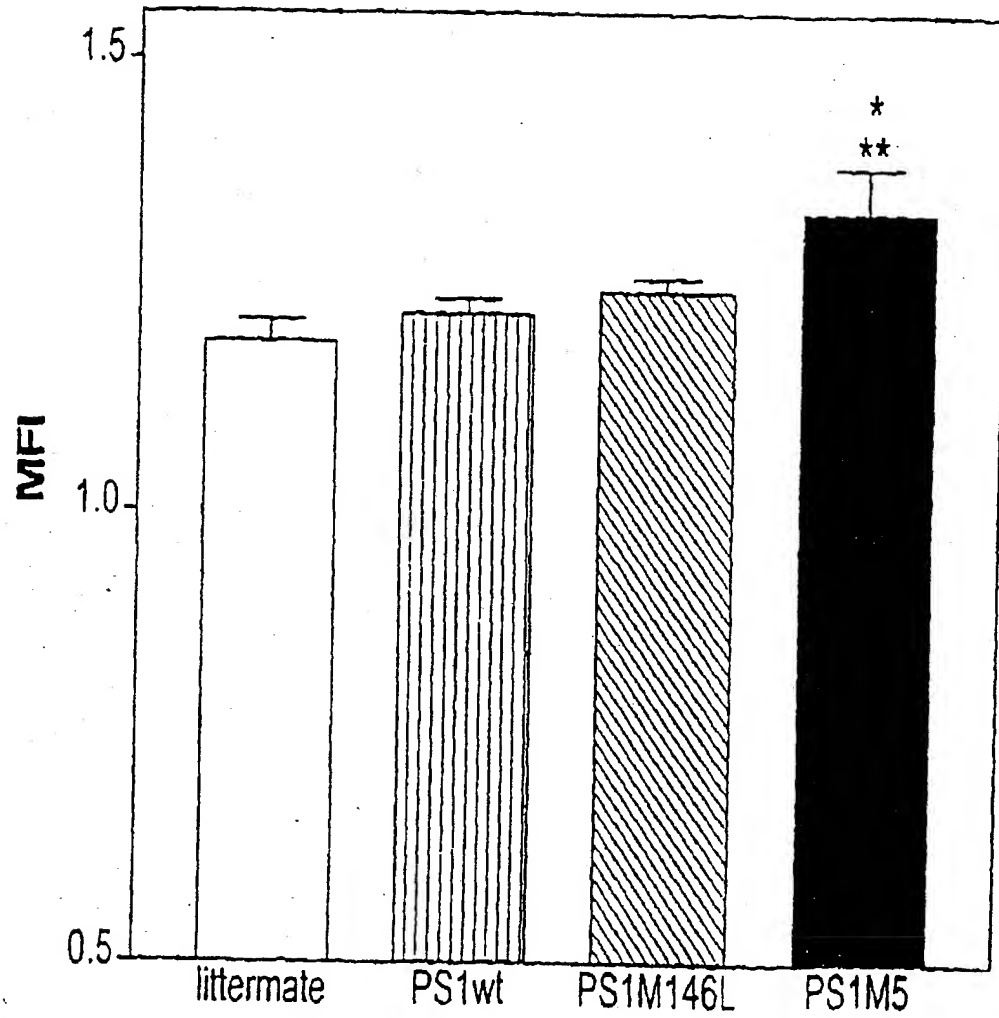


Figure 7

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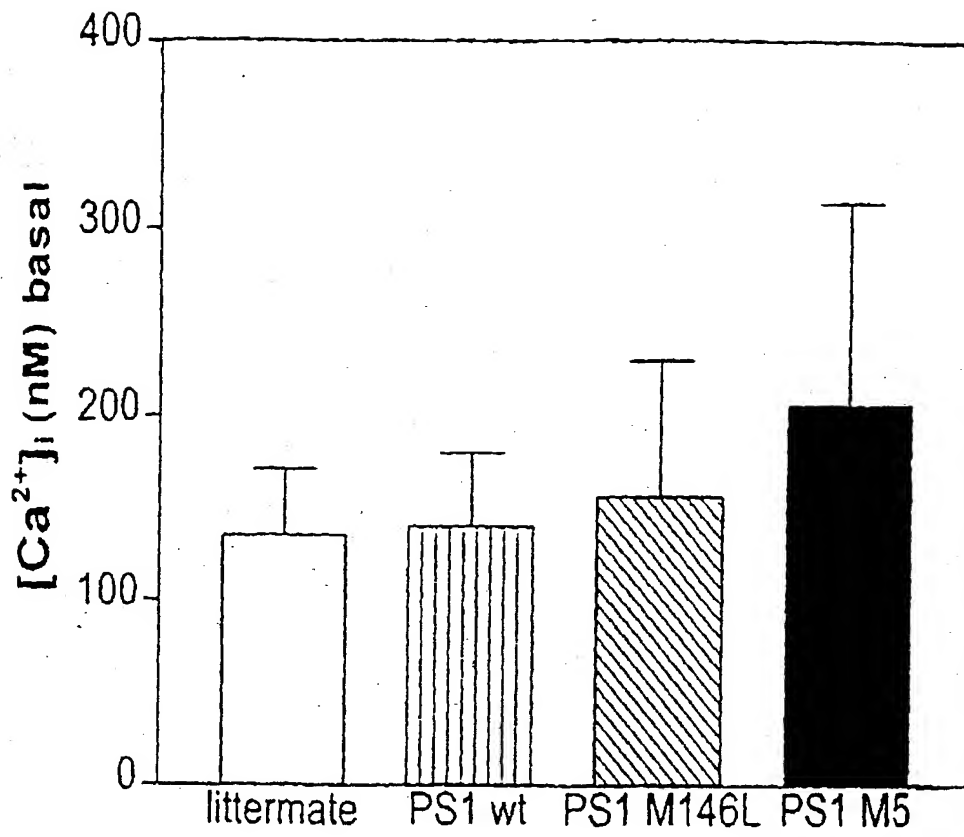


Figure 8

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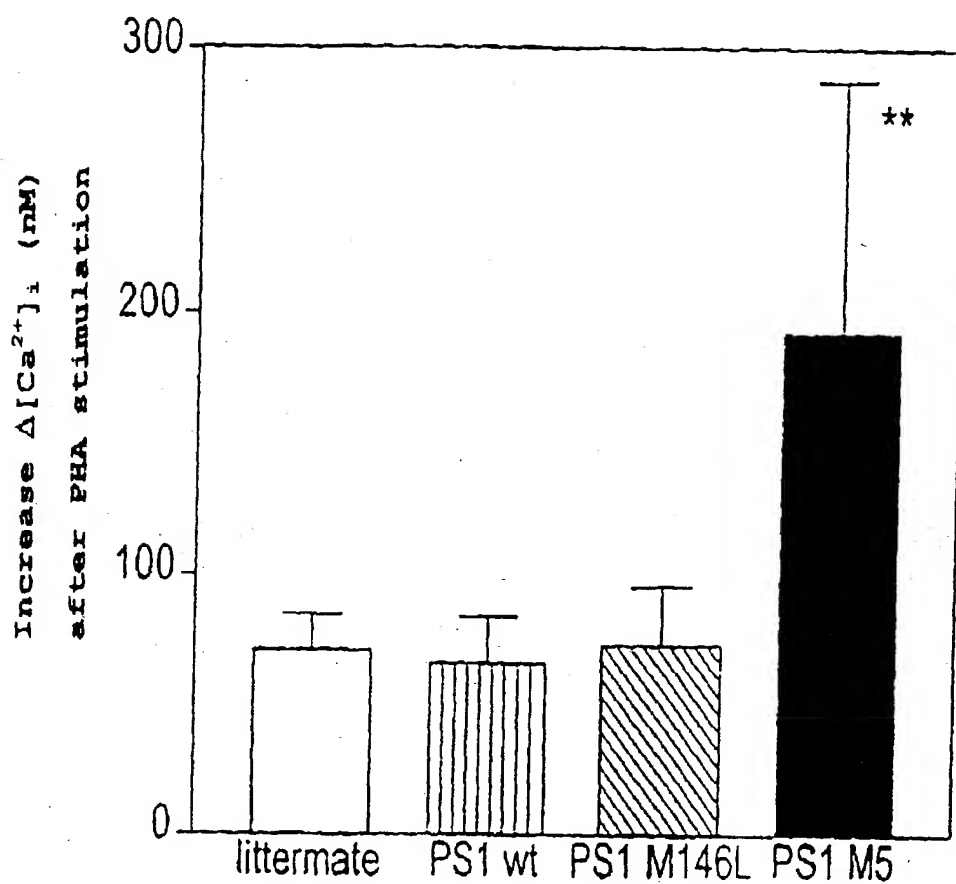


Figure 9

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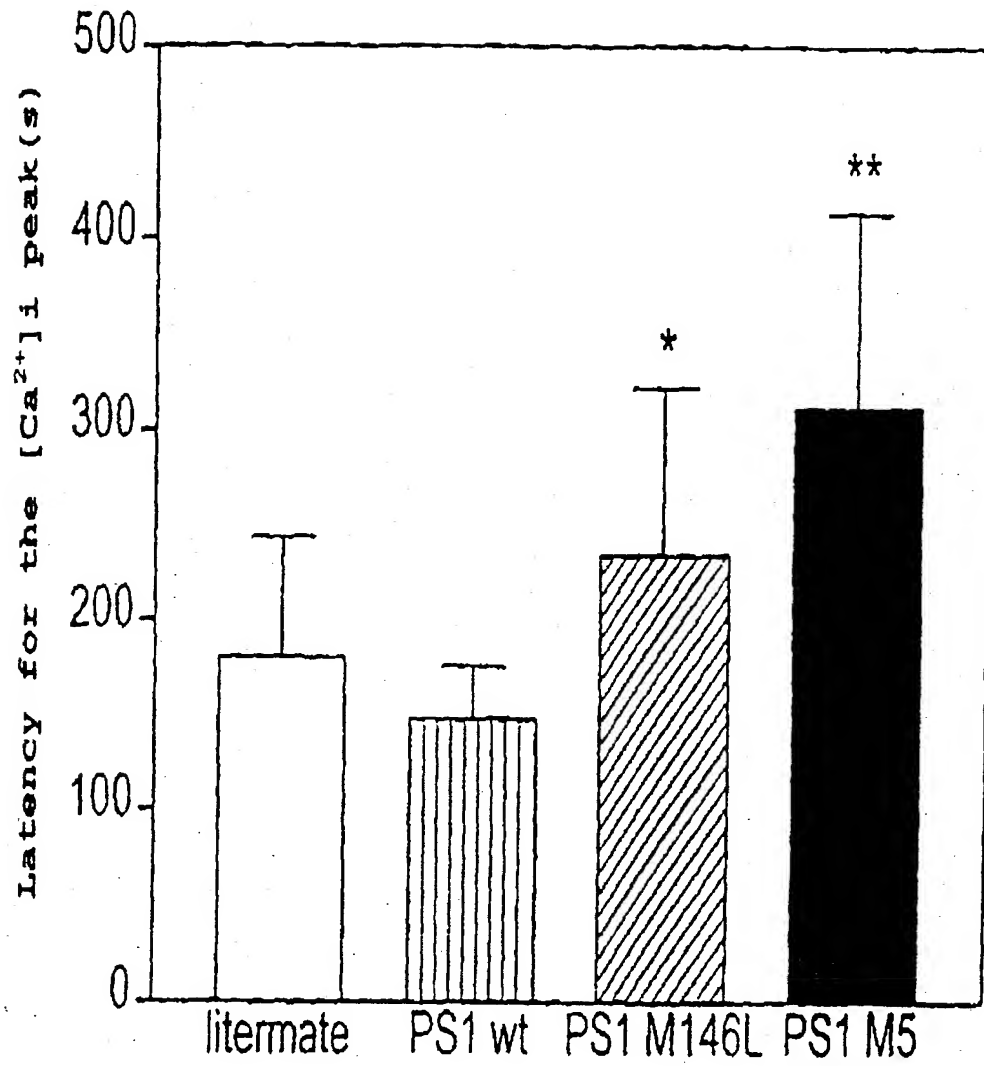


Figure 10

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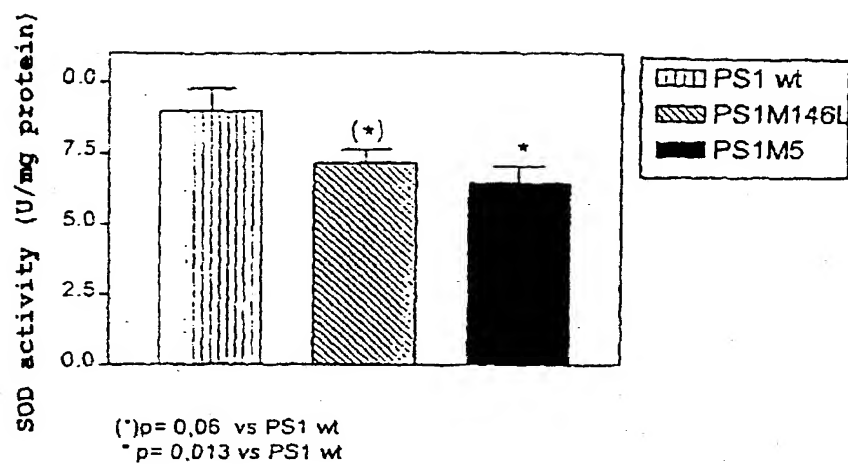
A

Figure 11A

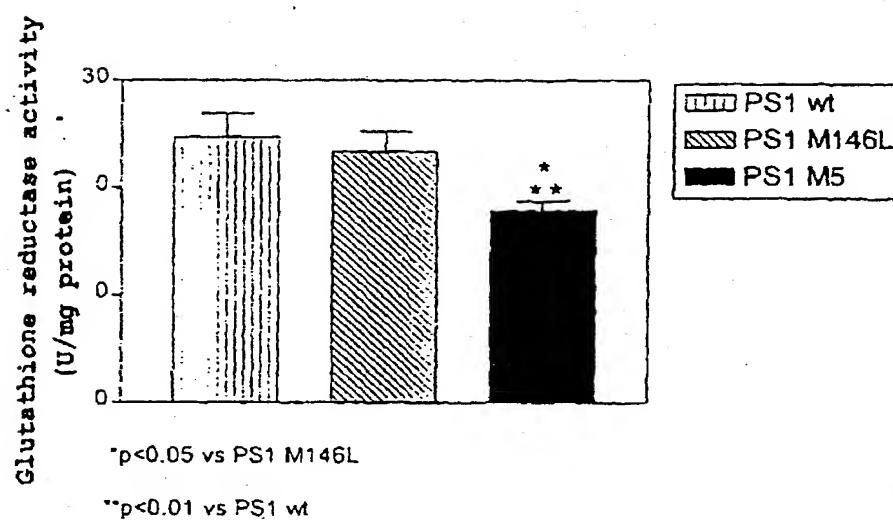
B

Figure 11B

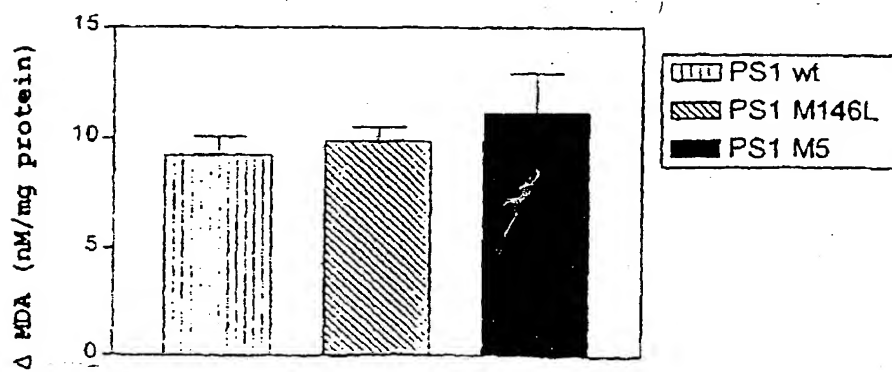
C

Figure 11C

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**DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN
APPLICATION DATA SHEET (37 CFR 1.76)**

As the below named inventor(s), I/we declare that:

This declaration is directed to:

- ☐ The attached application, or
☒ Application No. 10/088,139, filed on 15 March 2002,
☐ as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/ we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including material information which became available between the filing date of the prior application and the National or PCT International filing date of the continuation-in-part application, if applicable; and

All statements made herein of my/own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Anne Eckert

Signature: 22. 10. 2002 Anne Eckert

Citizen of: Germany

Inventor two: Walter Muller

Signature: _____

Citizen of: Germany

Inventor three: Christian Czech

Signature: _____

Citizen of: France

Inventor four: Laurent Pradier

Signature: _____

Citizen of: France

☒ Additional inventors are being named on 1 additional form(s) attached hereto.

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FULL NAME OF INVENTOR(S)Inventor one: Anne EckertSignature: _____ Citizen of: GermanyInventor two: Walter MullerSignature: Walt Muller Citizen of: Germany DEXInventor three: Christian CzechSignature: _____ Citizen of: FranceInventor four: Laurent PradierSignature: _____ Citizen of: France☒ Additional inventors are being named on 1 additional form(s) attached hereto.

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FULL NAME OF INVENTOR(S)

Inventor five: Gunter Tremp

Signature: *Gunter Tremp*

Citizen of: Germany

☐ Additional inventors are being named on _____ additional form(s) attached hereto.

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